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CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

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13. ABSTRACT *(Maximum 200 words)*

The adenovirus E1A oncoprotein sensitizes primary cells to undergo apoptosis following treatment with anticancer agents through a mechanism involving inactivation of the retinoblastoma (Rb) protein and stabilization of p53 tumor suppressor. Anticancer agents ultimately induce apoptosis by activating caspases, a family of cystein proteases that are essential components of the cell death machinery. Caspases are expressed as latent pro-enzymes and processed to active enzymes during apoptotic cell death. To further understand the mechanism whereby E1A promotes chemosensitivity, we examined pro-caspase levels in primary fibroblasts expressing E1A. Introduction of E1A greatly increased the levels of pro-caspases 2, 3, 7 and 8 through a post-transcriptional mechanism. The Rb binding domain of E1A is essential for pro-caspase induction, indicating that inactivation of Rb contributes to this effect. In contrast, the p53 and $p19^{ARF}$ tumor suppressors are dispensable for caspase induction, since E1A induces pro-caspase levels in p53-/- and ARF-/- MEFs. Nevertheless, the ability of E1A to induce pro-caspases and p53 may synergize to promote apoptosis. These studies provide a direct connection between a pro-apoptotic oncogene (E1A) and the cell death machinery. Caspase induction by E1A may contribute to its ability to promote chemosensitivity.

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T. O'Leary 7/25/99

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TABLES OF CONTENTS

INTRODUCTION

Apoptosis is a programmed form of cell death that plays an important role in malignancy by shifting the balance from tumor proliferation to its regression. Anticancer drugs act by activating apoptosis in tumor cells. Mutations in apoptotic pathways can lead to anticancer drug resistance and therefore can promote tumor progression. Our lab is working to elucidate the molecular mechanisms of apoptosis in oncogenically-transformed primary Mouse Embryo Fibroblasts (MEFs). We have chosen this model system because of the fact that it lacks mutations and alterations that are common to immortal cell lines; of the ability to use genetic approach to study apoptosis (availability of knock-out mouse lines); of the ease of gene manipulations (retroviral mediated gene transfer technique) in MEFs. The adenovirus E1A oncoprotein sensitizes primary cells to undergo apoptosis following treatment with anticancer agents. It was shown that E1A induced sensitivity in MEFs is similar to chemosensitivity of spontaneous tumors. We expect that further insight into mechanisms of programmed cell death in oncogenically-transformed MEFs will provide a fuller understanding of the role of apoptosis in real tumor progression such as breast cancer and will lead to the developing new strategies for anti-cancer therapy.

I am interested in determining the role of caspases in apoptosis in MEFs. Caspases are a family of cystein proteases that are expressed in latent pro-enzyme forms. Caspases are essential components of apoptotic machinery, and activation of pro-caspases is an obligatory step in the induction and execution of apoptosis triggered by many anticancer drugs. The goal of my thesis project is to identify caspases that are involved in programmed cell death in primary MEFs, to reveal caspases activated during distinct apoptotic programs, to determine the effect of E1A expression on caspases in the cells and finally to establish the role of one particular caspase in distinct forms of apoptosis in oncogenically-transformed MEFs.

BODY

Aim 1. Identification and characterization of distinct apoptotic pathways in E1A/ras-transformed MEFs.

Aim 1 was completed during previous year.

Aim 2. Identification of caspases activated during distinct apoptotic programs in ER/MEFs.

Identification of caspases expressed in wild-type E1A/ras/MEFs.

Nine members of mouse caspase family have been identified. I have established that Caspases-2,3,6,7,8 are expressed in primary MEFs at the RNA

level (fig. 1). Caspases-2,3,7,8 and 11 can also be detected at the protein level (fig. 2). Introduction of E1A oncogene greatly increased protein level of the unprocessed forms of the tested caspases. Moreover, caspase induction was p53 independent. I detected little, if any, induction of caspases at mRNA levels. That argues that this process might occur at the translational or post-translational levels. For example, E1A could somehow alter protein stability of pro-caspases.

E1A oncoprotein is known to sensitize cells to undergo apoptosis following treatment with anticancer agents through a mechanism involving inactivation of retinoblastoma (Rb) protein and stabilization of p53 tumor suppressor. The downstream events of this mechanism remain to be discovered. Anticancer agents ultimately induce apoptosis by activating caspases. Caspases are expressed as latent pro-enzymes and processed to active enzymes during apoptotic cell death. It is feasible that the high levels of pro-caspases that are available for activation by apoptotic agents could predispose cells to apoptosis. Caspase induction by E1A may be a part of the mechanism by which E1A promote chemosensitivity.

Since E1A promotes apoptosis and causes dramatic induction of pro-caspases I have focused my research on mechanism of pro-caspase induction by E1A (see Aim 4).

Generation of monoclonal antibodies against mouse caspases expressed in E1A/ras/MEFs.

This task was completed during previous year.

Establishing the pattern of caspase activation in wild-type E1A/ras/MEFs.

This task was completed during previous year.

Aim 3. Establishing the role of Caspase-2 in distinct forms of apoptosis in oncogenically transformed mouse embryo fibroblasts.

Aim 3 was completed during previous year.

Aim 4. Determination of the mechanism by which the E1A oncogene induces the pro-caspase levels.

My results from Northern blot analysis of caspase expression clearly showed that pro-caspase induction by E1A occured on post-transcriptional level (fig. 1). Moreover this induction was independent on p53 function since E1A oncogene still induced pro-caspases in p53-/- cells (fig. 2).

Determination of E1A protein regions responsible for caspase induction.

I used several E1A deletion mutants that are known to bind certain cellular factors (fig. 3). I introduced these mutants to wild type MEFs and by Western blot analysis with anti-caspase antibodies I have established that the CR2 region

of E1A and possibly the N-terminal region are responsible for caspase upregulation (fig. 4). CR2 region of E1A is known to bind members of Rb family of pocket proteins. For all the pro-caspases that were tested deltaCR2 mutant of E1A was unable to induce pro-caspase levels. To prove that observed effect was not species-specific, I have shown that CR2 region of E1A was responsible for caspase induction in human fibroblasts (IMR90) (fig. 4). The N-terminal region of E1A is known to bind p300 protein. I have determined that this region was important for Caspase-7 induction both in mouse and human fibroblasts. (fig. 4)

Establishing the role of Rb in regulation of the pro-caspase levels.

Since CR2 region of E1A can to bind such pocket proteins as Rb, p107, p130 and Rb is required for chemosensitization of MEFs, I decided to determine whether one of the functions of Rb in the normal cell is to block pro-caspase induction.

I compared pro-caspase levels between wild type MEFs and Rb-/- MEFs. Expression of caspases in Rb-/- cells was increased compared to wild type MEFs (Fig. 5). For several caspases these levels were comparable to levels that were observed upon introduction of wild type E1A. For others, these levels were lower (data not shown). Nevertheless, these results clearly showed that Rb blocked pro-caspase expression in primary cells. Also these results argue that Rb may synergize with other cellular protein(s) possibly p300 that is known to bind the N-terminal region of E1A to repress pro-caspases.

To prove that it was Rb that was responsible for pro-caspase induction, I introduced all the above E1A mutants into Rb-/- MEFs with the expectation that in this background the deltaCR2 E1A mutant will not have any defect in caspase upregulation. For all tested caspases the results were like expected (fig. 6).

Next, I reintroduced Rb back into Rb-/- cells and proved that pro-caspase levels have diminished. I accomplished this by utilizing temperature-sensitive human Rb mutants (Tiemann, F. et al). Osteocarcoma cell lines (ts1 and ts2) that stabily express tsRb were used in this experiment. I grew these cells at permissive and non-permissive temperatures. Rb protein was not expressed at non-permissive temperature. I monitored the suppression of pro-caspase induction by Western blot analysis with anti-caspase antibodies (fig. 7). I used two osteocarcoma cell lines U2OS (Rb positive) and SAOS 2 (Rb negative) as controls. All these data together clearly demonstrated that induction of pro-caspases in the cells by E1A occured mostly by inactivation of Rb protein and that one of the function of Rb was to block pro-caspase upregulation.

To determine whether pro-caspase induction alone could lead to chemosensitivity I treated wt MEFs, Rb-/-MEFs, wt E1A, Rb-/- E1A with adriamycin, an anticancer agent. Apoptosis was scored by Trypan blue exclusion and DAPI (fig. 8). I have shown that increased levels of pro-caspases could stimulate chemosensitivity (compare wt MEFs and Rb-/-MEFs), but alone were not sufficient for complete chemosensitivity (compare Rb-/-MEFs and wt E1A).

Determination of the level at which Rb-dependent pro-caspase suppression occurs.

A. Transcription.

From the Northern blot analysis I have already established that mRNA levels of the caspases were practically the same in primary and E1A infected cells. This observation argues that Rb-dependent regulation occurs at the protein level.

B. Stability.

It is known that E1A stabilizes p53 protein levels, in part by inactivating Rb. Therefore, I predict that E1A would also stabilize pro-caspases. To determine this I plan to perform the following series of experiments.

- I will measure caspase turnover in primary MEFs and E1A infected MEFs by a pulse chase experiment.
- Next, I plan to use proteasome specific inhibitors on wt primary MEFs to see the possible induction of caspase levels (Dick L. et al).
- I will take advantage of ts cell line defective in the ubiquitin pathway to look for induction of caspase levels at non-permissive temperature (Chowdary D. et al).

C. Translation.

In the case when I would not be able to detect pro-caspase regulation on protein stability level I plan to look for the possibility of regulation on the level of translation.

- I will use translation efficiency assay (polysome gradient) to measure the levels of caspases translation (Mullner E.W. et al, Fagan R.J. et al).

Identification of the Rb partners acting in Rb-dependent pathway of suppression of the pro-caspase levels.

Many cellular proteins have been identified to interact with Rb. Among them are E2F1, Mdm2, Cyclin D. Other proteins were shown to modulate Rb activity – p16, p21. P19 was recently shown to promote Mdm2 degradation and stabilization of p53 in the cells. I took advantage of available knock-out mouse strains to determine which of those molecules participate in stabilization (destabilization) of pro-caspases.

I compared the ability of E1A to induce pro-caspase levels in wild type and E2F1-/-; p19-/-, p16-/- MEFs (fig. 9). My data showed that Rb-dependent pro-caspase suppression did not require E2F1, p16 and p19.

Overexpression of E2F1 alone was sufficient to induce apoptosis in E1A/MEFs.I overexpressed E2F1 in wt MEFs and in p53-/-MEFs to determine the ability of this protein to induce pro-caspase levels (fig. 10). Since E2F1 induced massive apoptosis in wt MEFs, I looked for the effect of E2F1

overexpression in p53-/-MEFs (I already have established that pro-caspase induction was p53 independent). My results argue that overexpression of E2F1 transcription factor is sufficient to induce pro-caspase levels. Since my previous results showed precisely that it was not E2F1 itself that was responsible for pro-caspase induction other member of E2F family possibly could do this job.

Mdm2 is playing an important role in regulation of p53 levels in the cells. It was shown that Mdm2 can possibly bind Rb. Mdm2 is one of the molecules that are cleaved during early stages of apoptosis by caspases. To determine the role of Mdm2 in pro-caspase regulation I overexpressed Mdm2 in wt E1A MEFs and in p53-/-MEFs to look for possible suppression of caspase induction and in wt MEFs to look for pro-caspase induction (fig. 11). My data showed none of these effects.

The results obtained in Aim2 and Aim4 provided the evidence that links apoptotic cell machinery with ability of E1A to sensitize cells to apoptosis. The molecular mechanisms of E1A promoted chemosensitivity remains poorly understood. Such events as inactivation of the retinoblastoma protein, release of E2F's transcription factors and stabilization of p53 through p19 were shown to be important but not sufficient for the full chemosensitivity. My work provided an evidence for another possible mechanism of E1A-induced E2F's mediated chemosensitivity that can be accomplished by the synergy of pro-caspase induction with p53 stabilization (fig. 12).

APPENDICES

Key research accomplishments

1. E1A induces pro-caspases on the protein level
2. This induction is p53-independent
3. This induction occurs mostly through the inactivation of Rb protein
4. Rb blocks pro-caspase induction
5. Increased levels of pro-caspases can stimulate chemosensitivity
6. Increased levels of pro-caspases alone are not sufficient for complete chemosensitivity
7. Rb-dependent pro-caspase suppression does not require E2F1, p16 and p19
8. Overexpression of Mdm2 and Bcl2 oncogenes does not alter pro-caspase levels
9. Overexpression of E2F1 transcription factor induces pro-caspases

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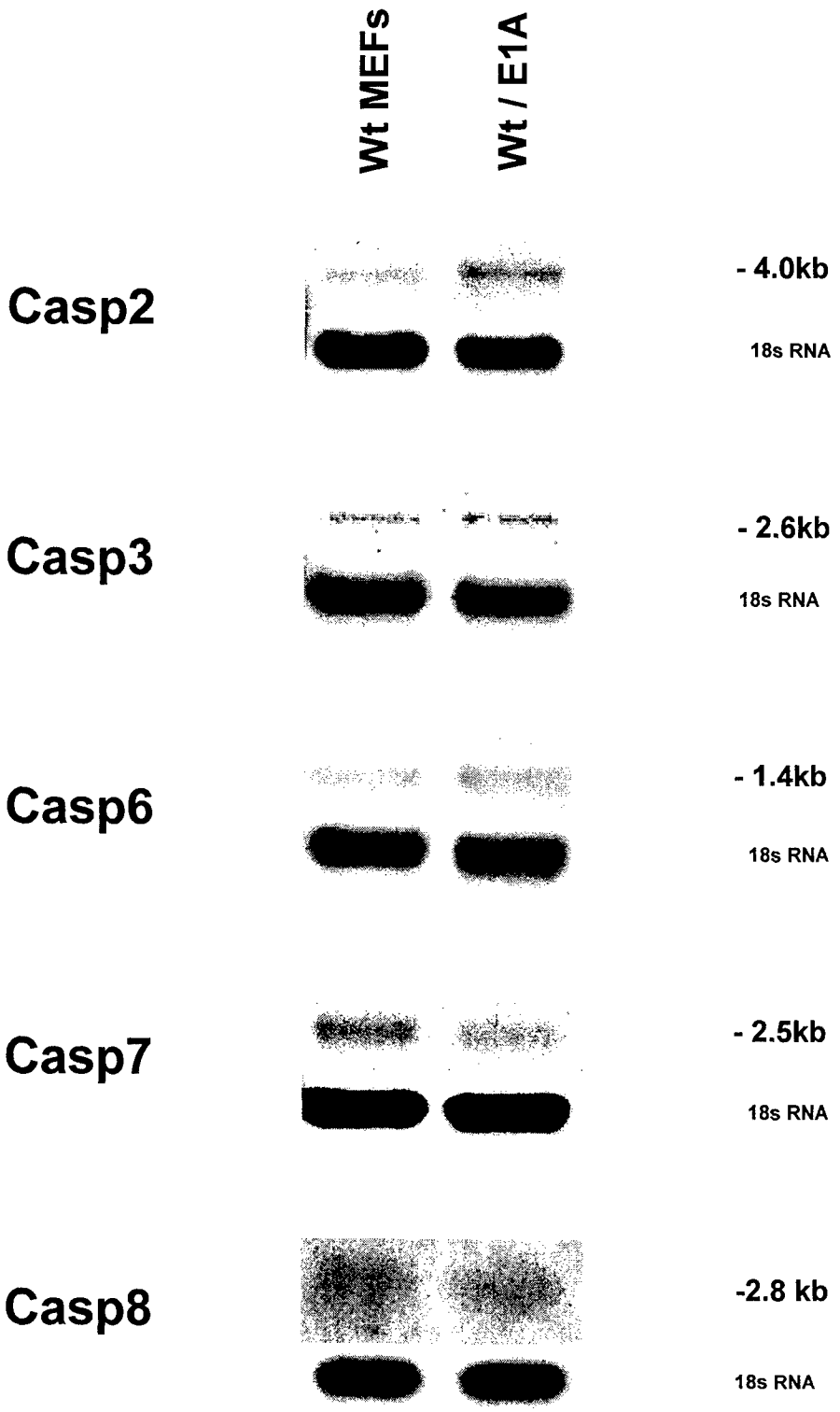
Mullner, E.W., Neupert, B., and Kuhn, L.C. (1989) A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. Cell 58, 373-382.

Fagan, R.J., Lazaris-Karatzas, A., Sonenberg, N., and Rozen, R. Translational control of ornithine aminotransferase. J Biol Chem 266, 16518-16523.

Fig. 1

Northern blot analysis of caspase expression in primary MEFs vs. E1A infected MEFs



Western blot analysis of caspase expression in untreated MEFs

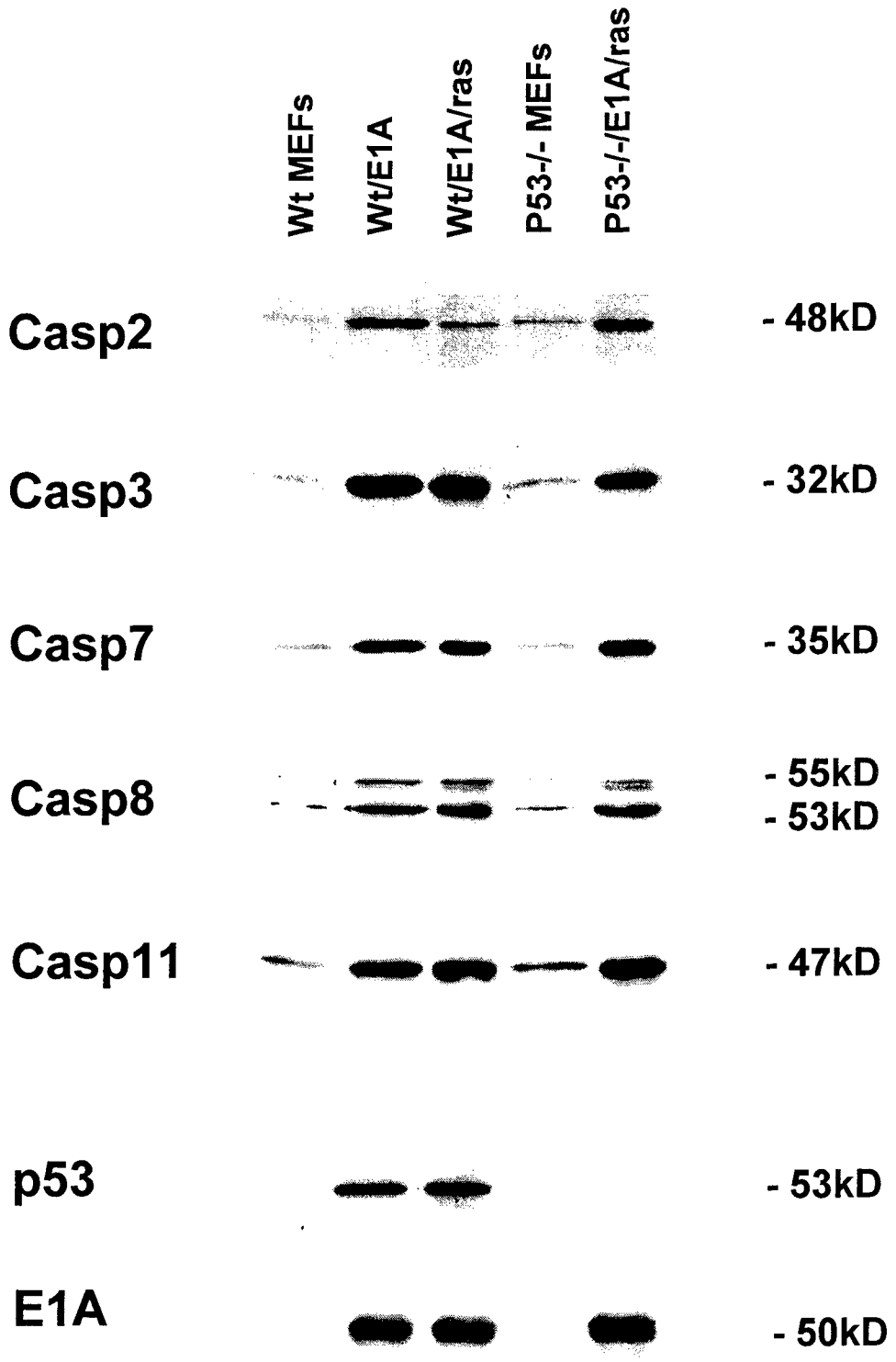
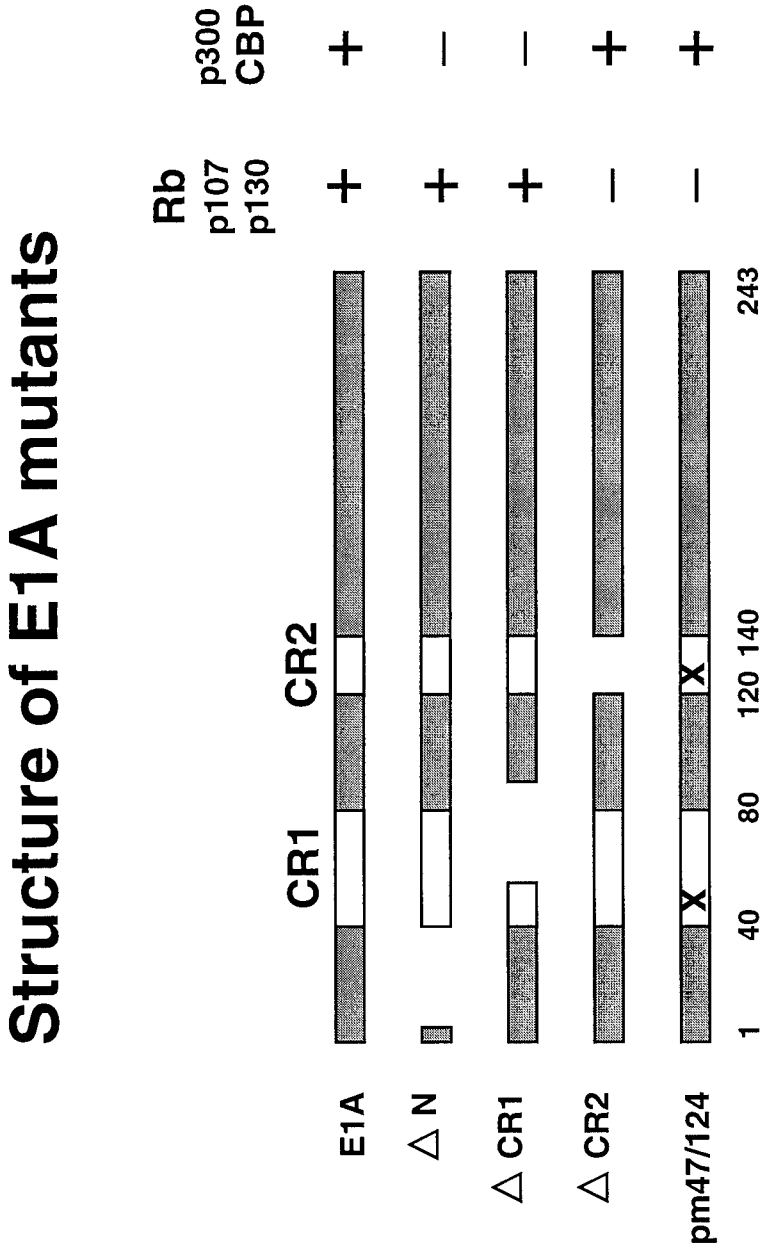


Fig. 3


Structure of E1A mutants
Rb
p107
p130
p300
CBP
CR1
CR2
E1A
+
+
Δ N
+
−
Δ CR1
+
−
Δ CR2
−
+
pm47/124
−
+
1
40
80
120
140
243
X
X

CR2 region of E1A is required for pro-caspase induction in wild-type primary cells

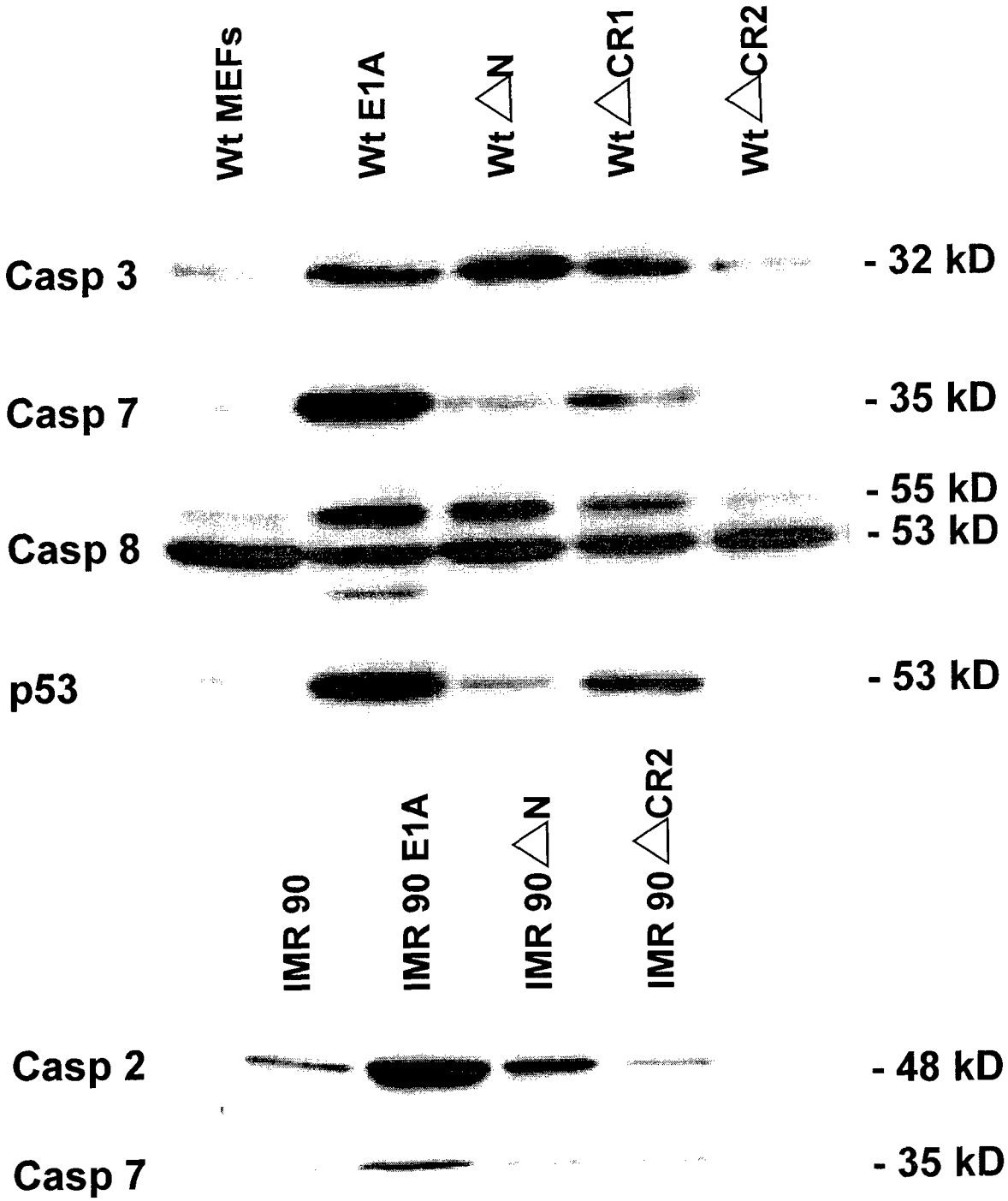


Fig. 5

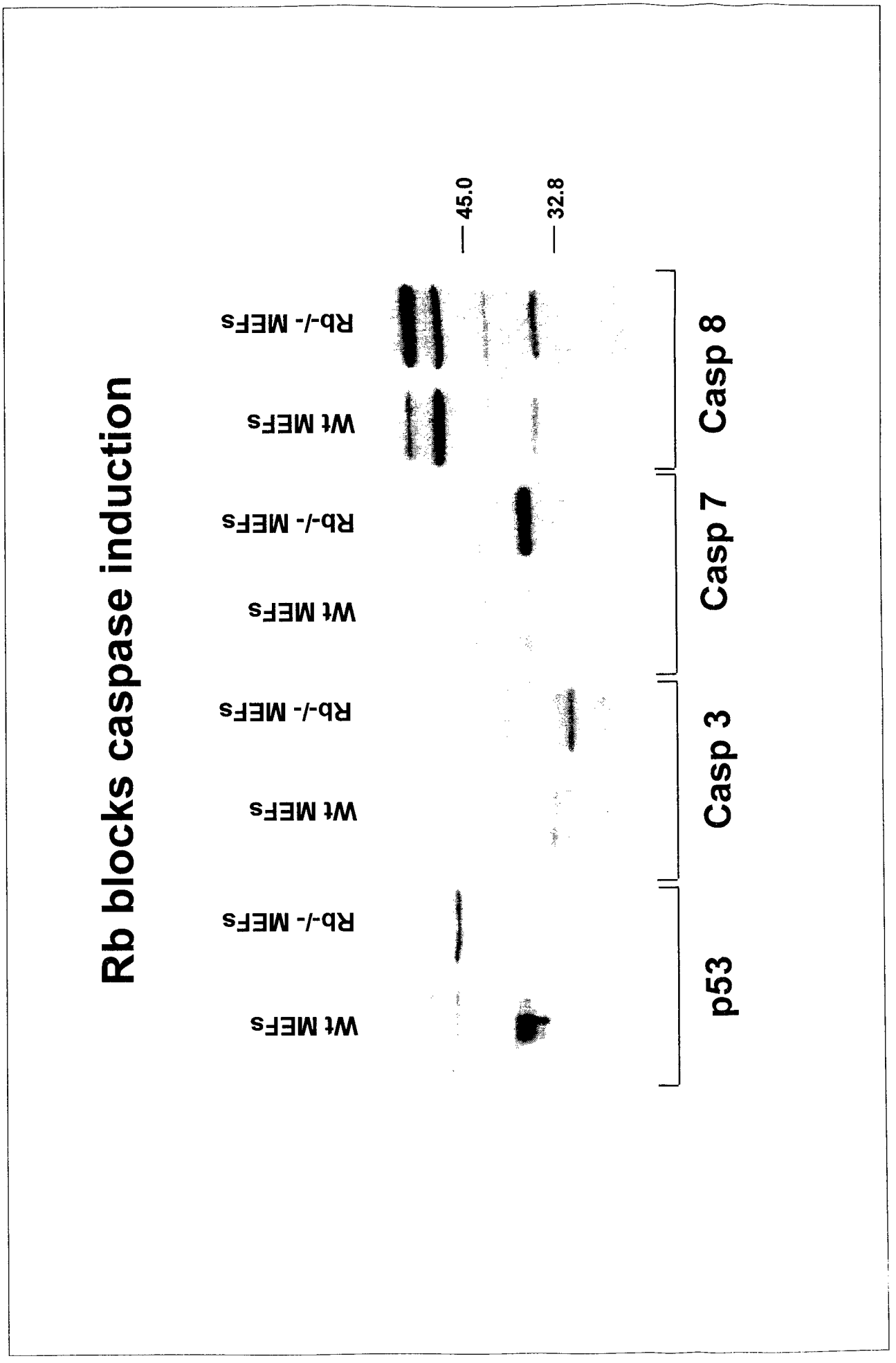
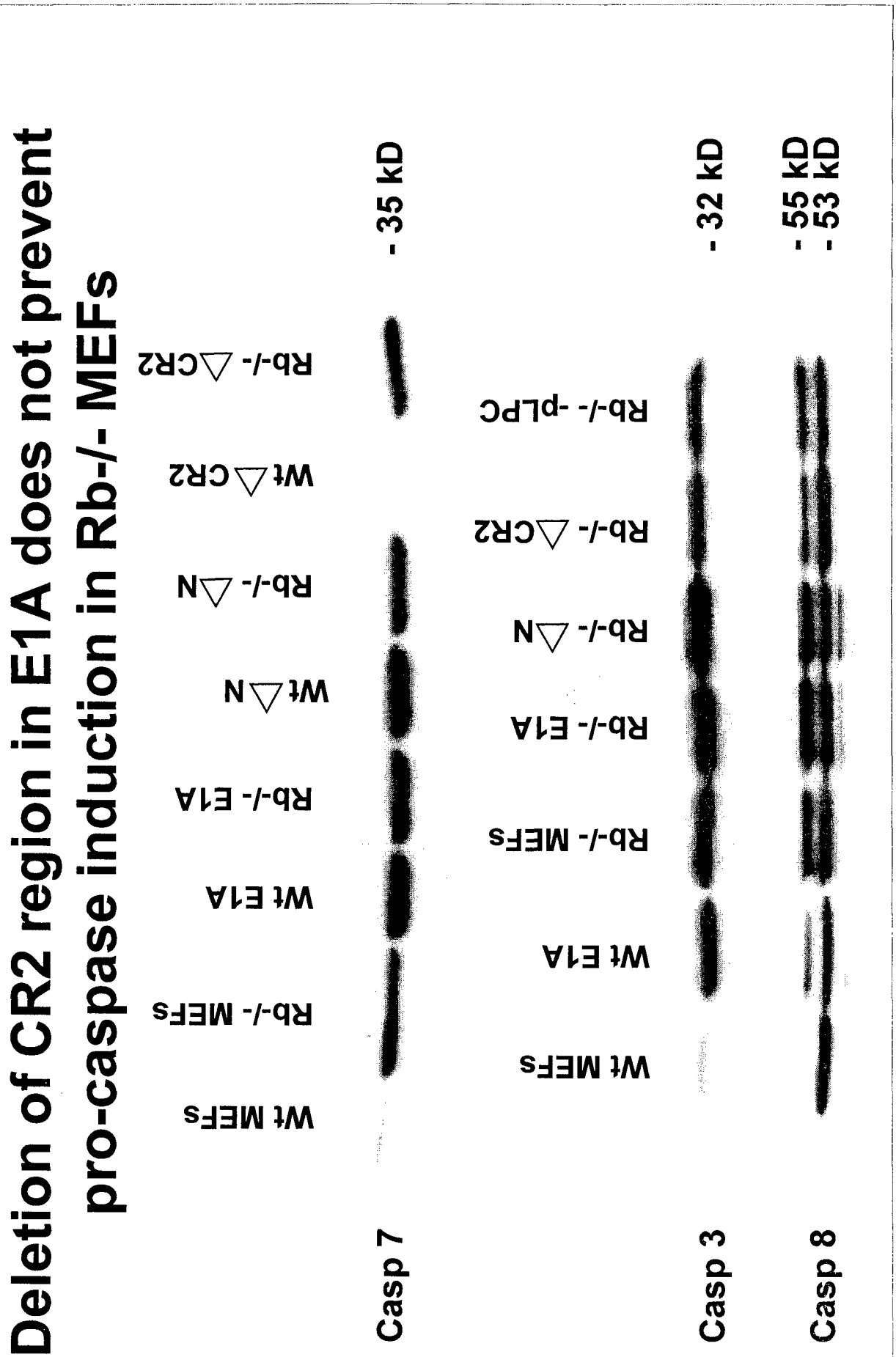

Rb blocks caspase induction
45.0
32.8
Rb-/- MEFs
Wt MEFs
Rb-/- MEFs
Wt MEFs
Rb-/- MEFs
Wt MEFs
Rb-/- MEFs
Wt MEFs
p53
Casp 3
Casp 7
Casp 8

Fig. 6


Deletion of CR2 region in E1A does not prevent pro-caspase induction in Rb-/- MEFs
Wt MEFs
Rb-/- MEFs
Wt E1A
Rb-/- E1A
Wt ΔN
Rb-/- ΔN
Wt ΔCR2
Rb-/- ΔCR2
Casp 7
- 35 kD
Wt MEFs
Wt E1A
Rb-/- MEFs
Rb-/- E1A
Rb-/- ΔN
Rb-/- ΔCR2
Rb-/- -pLPC
Casp 3
- 32 kD
Casp 8
- 55 kD
- 53 kD

Inactivation of a temperature-sensitive Rb protein induces pro-Caspase 7 levels in osteosarcoma cell line SAOS 2

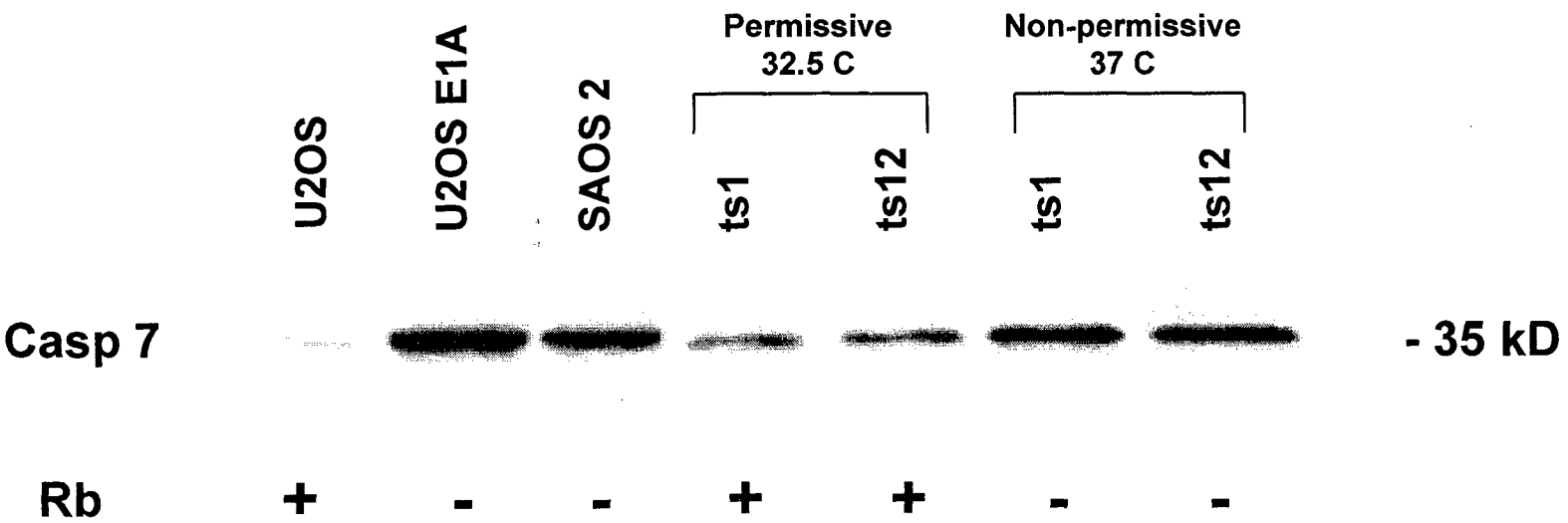


Fig. 7

Fig. 8

Induction of pro-caspases is not sufficient for chemosensitivity induced by E1A

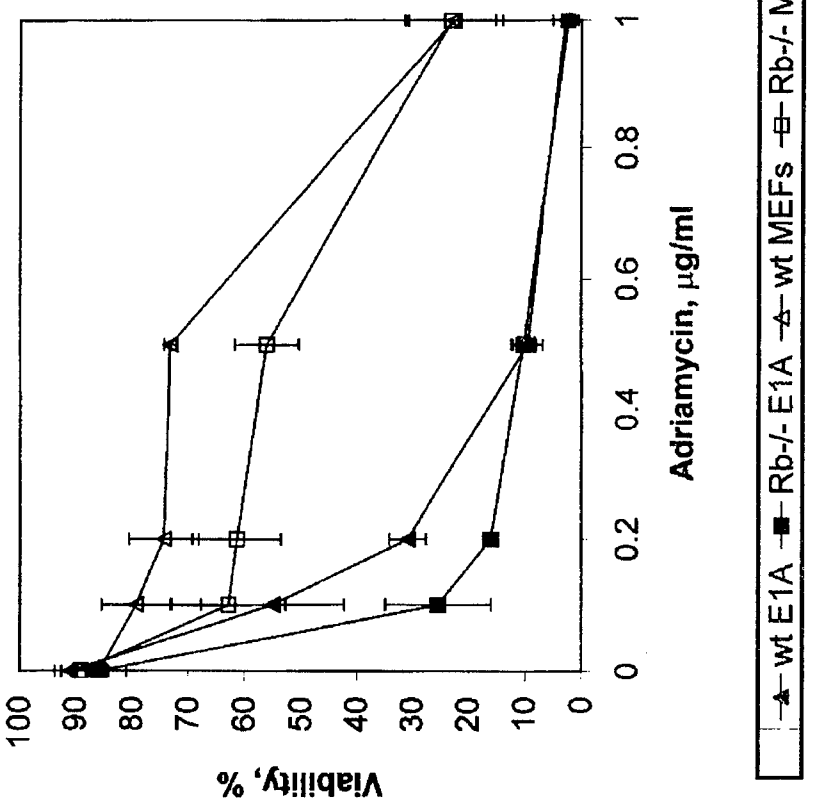


Fig. 9

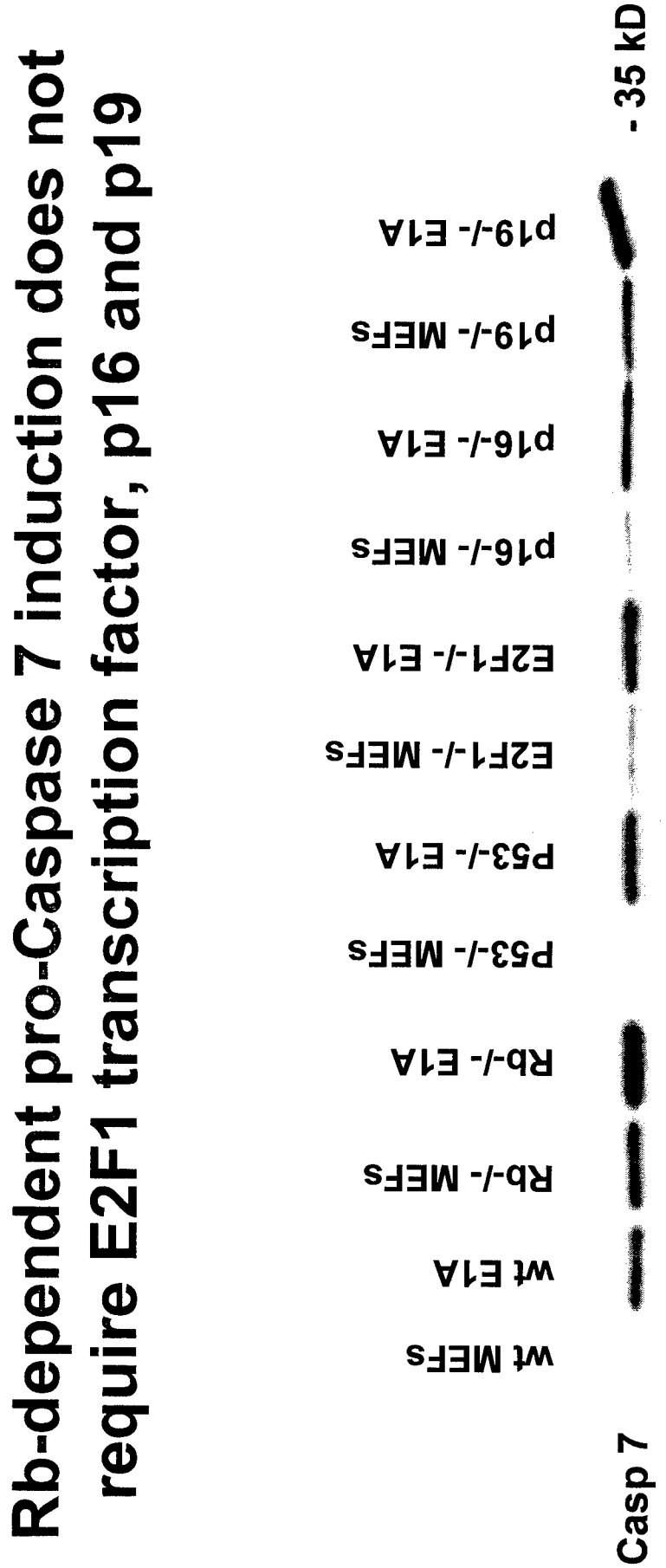

Rb-dependent pro-Caspase 7 induction does not require E2F1 transcription factor, p16 and p19
- 35 kD
Casp 7
wt MEFs
wt E1A
Rb-/- MEFs
Rb-/- E1A
P53-/- MEFs
P53-/- E1A
E2F1-/- MEFs
E2F1-/- E1A
p16-/- MEFs
p16-/- E1A
p19-/- MEFs
p19-/- E1A

Fig. 10

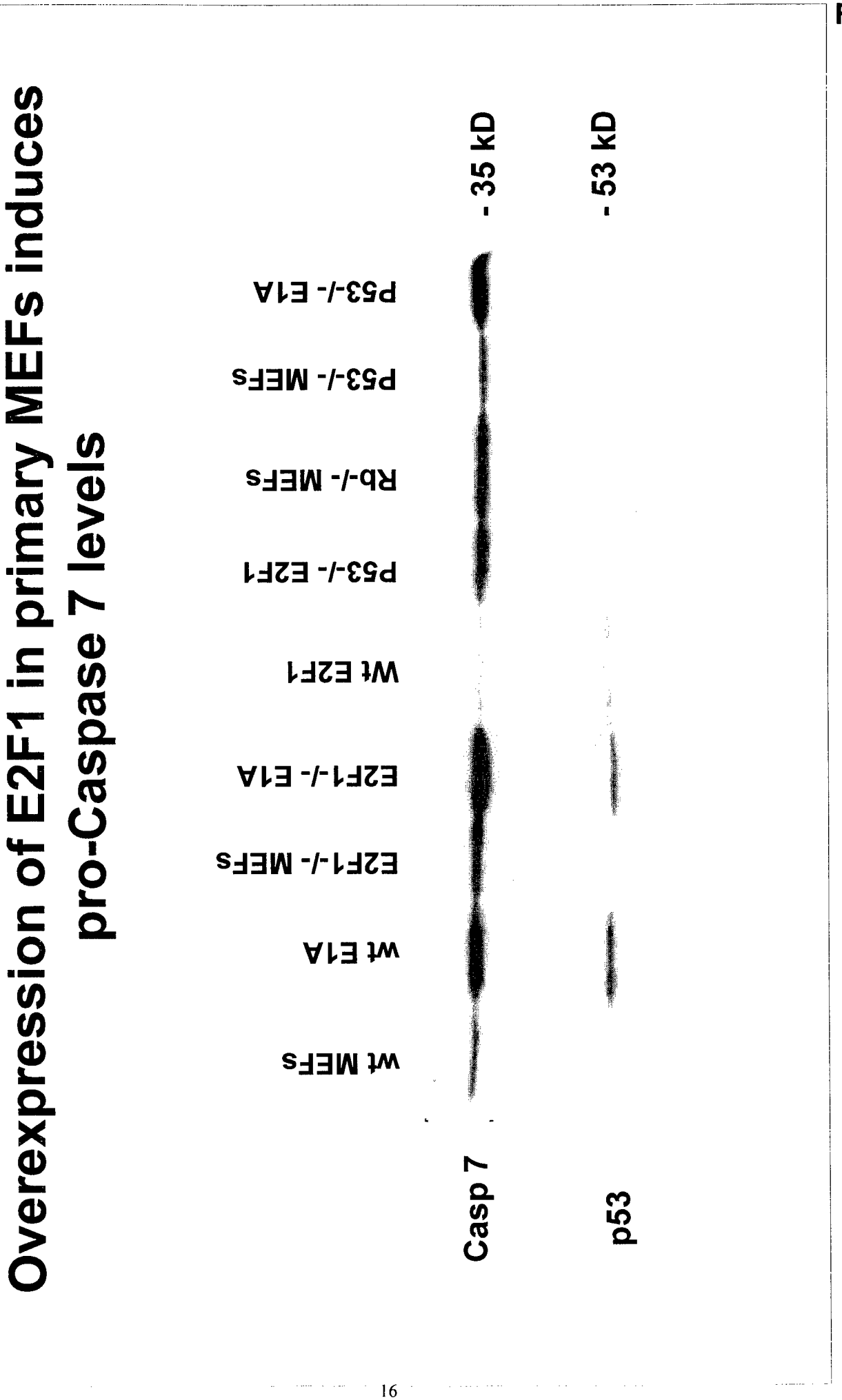

Overexpression of E2F1 in primary MEFs induces pro-Caspase 7 levels
- 35 kD
- 53 kD
P53-/- E1A
P53-/- MEFs
Rb-/- MEFs
P53-/- E2F1
Wt E2F1
E2F1-/- E1A
E2F1-/- MEFs
wt E1A
wt MEFs
Casp 7
p53

Fig. 11

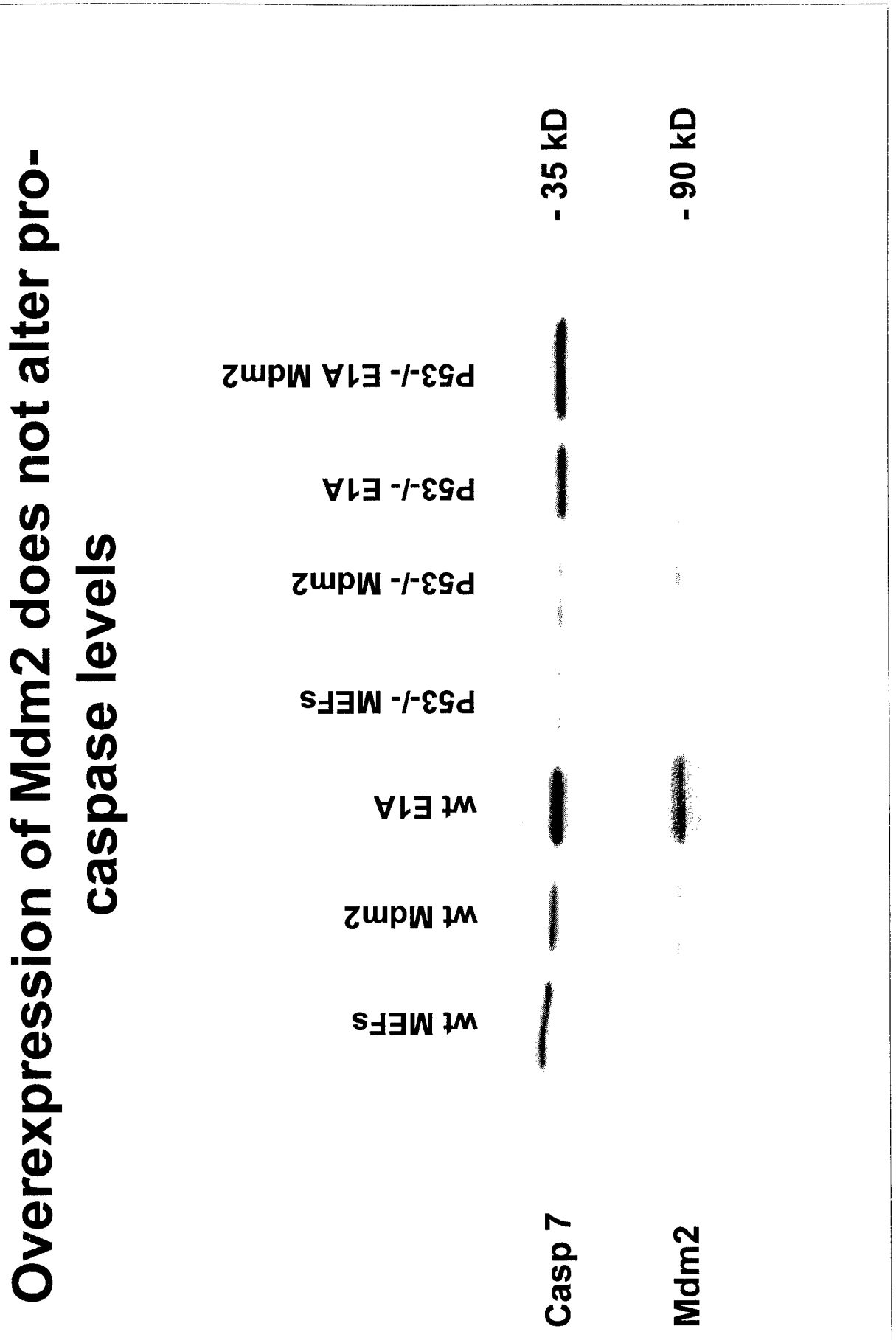
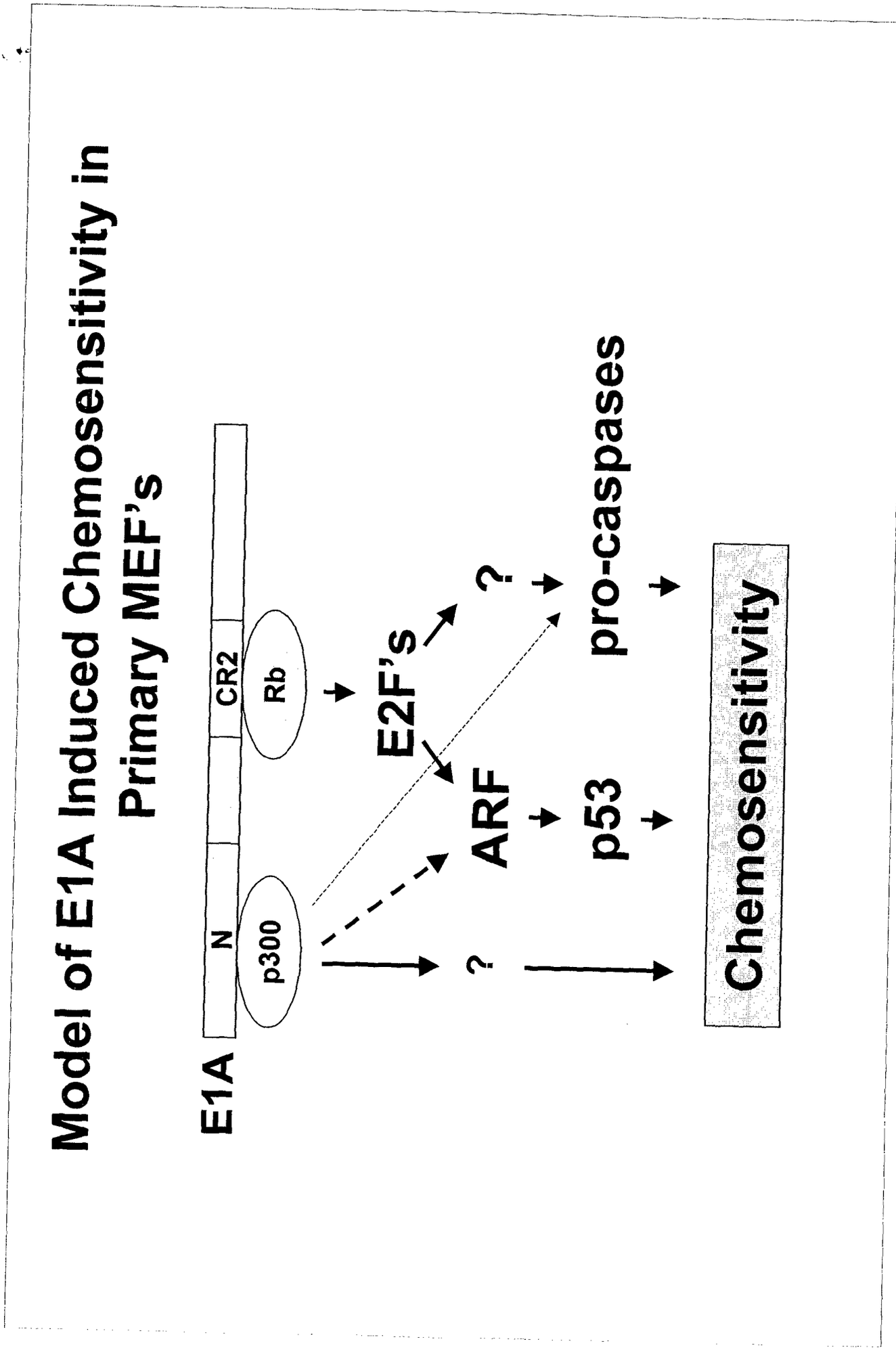

Overexpression of Mdm2 does not alter pro-caspase levels
Casp 7
Mdm2
- 35 kD
- 90 kD
wt MEFs
wt Mdm2
wt E1A
P53-/- MEFs
P53-/- Mdm2
P53-/- E1A
P53-/- E1A Mdm2

Fig. 12


Model of E1A Induced Chemosensitivity in Primary MEF's
E1A
N
CR2
p300
Rb
E2F's
?
ARF
p53
pro-caspases
?
Chemosensitivity

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